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BIOLOGICAL BULLETIN

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CONTENTS

		PAGE
GLASER, OTTO.	*Fertilization and Egg-secretions*	63
BODINE, JOSEPH HALL.	*Hydrogen ion Concentration of Protozoan Cultures*	73
CHILD, C. M.	*The Axial Gradients in Hydrozoa* . . .	78
CROZIER, W. J.	*Notes on Some Problems of Adaptation:*	
	4. *The Photic Sensitivity of Ogilbia* .	98
	5. *The Phototropism of Lima*	102
	6. *Relation of Light to the Pigmentation of Ascidians*	106
	7. *Regarding the Pigmentation of Stichopus moebii*	112
	8. *Concerning "Memory" in Actinians*	117

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BIOLOGICAL BULLETIN

FERTILIZATION AND EGG-SECRETIONS.[1]

OTTO GLASER,

AMHERST COLLEGE.

I.

The newer work on fertilization is directly traceable to the investigations of Frank R. Lillie.[2] It was he who first drew our attention to egg-exudates: later, the loose ends of observation and experiment united, in his mind, into a scheme whose novelty has played the part of an intellectual ferment. If we can ask new questions and for that matter, answer them too, more precisely than we could in 1913, this is largely due to the catalytic effects of the fertilizin hypothesis.

From a large list of besetting problems—most of them only solved in part—I shall select for discussion merely those that have interested me particularly, and first among these is the question whether egg-secretions have anything whatever to do with fertilization.

Skeptics, now and again, have been caught coquetting with the inevitable difficulty that egg-secretions can be discovered and analyzed in only one kind of egg at a time. By insisting on this point they appear to attach comparatively little value to the distinctions between prevalence and importance. However, we are no longer terrorized by one who brandishes the single case. Today, exudates are known not only from the eggs of one echinoderm and one annelid, but from every one of the ten species of echinoderms that have been tested. Not merely this, but additional cases have been reported from annelids, molluscs and tunicates. To the list of previously recognized instances,

[1] Read before the American Society of Zoölogists at a symposium on Fertilization, held in Chicago, December 30, 1920.

[2] An excellent summary of all the investigations in this field is to be found in Lillie's "Problems of Fertilization," University of Chicago Press, 1919.

I wish to add, parenthetically, another mollusc, the oyster, and two vertebrates, the fish, *Fundulus heteroclitus*, and the frog, *Rana pipiens*.

Though we may derive comfort from a distribution so varied, this in itself is no proof that the exudates are part and parcel of the mechanism of fertilization. Moreover the original method of demonstrating their importance is not free from suspicion. To wash eggs until all traces of their exudates have disappeared takes time; in the 18 to 36 hours necessary to accomplish this, the eggs themselves may undergo serious deterioration. Their failure therefore to develop if inseminated after washing cannot be attributed offhand to the removal of the exudates. Yet the original inference is correct[1] for the time required to remove the exudates may be cut in half by the use of running sea-water. By the use of charcoal[2] eggs may be freed from secretion completely in 3 to 4 hours; or, if the chorion is first removed, in 30 minutes. This falls well within the time limit within which unwashed eggs show no impairment of fertility. More convincing proof comes from experiments in which the fertility of eggs partially sterilized by the removal of exudate is measurably increased by the addition of freshly prepared secretion. Such an experiment with *Asterias* eggs was first carried out by Miss Woodward. Since then I have had an opportunity to verify her results on the eggs of *Echinarachnius parma*. These in a series of experiments were incompletely sterilized by the partial removal of the exudate. In one of the experiments the eggs were then divided into two lots; one of these was inseminated at once and several hours after fell into two groups—59 per cent. absolutely inactive ones and 41 per cent. in which development was going on. The latter group, however, was in turn divisible into cleavages patently anomalous and cleavages not distinguishable from the normal, approximately, in the ratio of 2 : 1. The

[1] There is one reservation; complete removal seems to have certain irreversible consequences. I have referred to the matter before. See this journal, Vol. XXVI., p. 395.

[2] The use of charcoal in this connection was suggested by Dr. G. H. A. Clowes. The suggestion was based on preliminary indications of the presence of enzymes and on the well-known efficacy of charcoal in removing these from solution. See Euler-Pope, "General Chemistry of Enzymes," p. 81.

second lot of eggs was treated with normal egg-secretion and inseminated in its presence. The results show a decrease in the inactive eggs to 50 per cent.—a decrease in the anomalous cleavages to 14 per cent. and an increase in normal divisions to 36 per cent.

II.

Our second question is, how do the exudates act? Naturally, we must formulate our explanation in terms of material entities. This, indeed, was done from the first, but the entity postulated was little more than a symbol for the observed effects.

These fall into two groups: the effects on spermatozoa and the effects on eggs. As Lillie first found, the spermatozoa are activated and exhibit a remarkable process of swarming and agglutination; the eggs, as I found, upon exposure to secretion develop spontaneously. Lillie attributed both phenomena to a single substance—the agglutinin of his earlier papers—the fertilizin of his later writings.

Lillie has introduced prevention as an aid to analysis, and has described two natural inhibitors. In the presence of one of these, the "anti-fertilizin" derived from the eggs themselves, spermatozoa do not swarm and agglutinate and eggs cannot be fertilized; in the presence of the other, contained in species-true blood, although the spermatozoa agglutinate, nevertheless the eggs fail to develop.

To account for these facts, Lillie made the following assumptions: The agglutinin has two bonds. One of these normally combines with a sperm-borne valence. The symptom of such union is the agglutination reaction. The other bond of the agglutinin unites with an egg-borne valence. The symptom of this union is the initiation of development. "Anti-fertilizin," prevents fertilization because it binds the spermophile group of the agglutinin and so renders the normal union with the sperm-valence impossible. Inasmuch as the inhibitor in the blood does not affect the agglutination reaction, this substance is assumed to occupy the ovophile bond of the agglutinin. Applying the picture-language of Ehrlich, Lillie called the agglutinin an "amboceptor," whose two bonds are satisfied in fertilization in the suggested manner.

The idea of attempting to isolate Lillie's amboceptor appeared foolhardy to many of my friends. As it was my own early attempts showed merely how it could not be done and what not to look for. The first ray of positive light came from the experiments of Richards and Miss Woodward with X-rays ('15). Their results suggested that the amboceptor might be an enzyme, and, as a matter of fact, by adapting a method previously employed by Robertson ('12) for the removal of oöcytase from ox blood, Miss Woodward succeeded in isolating a definite substance from the secretions of arbacia eggs ('18).

The substance in question was gotten in the form of a white powder easily soluble in sea water and in fresh. It did not cause the characteristic swarming and agglutination of the spermatozoa. It did however have decided virtues as a parthenogenetic agent. Moreover, it appeared to possess lipolytic properties. At any rate, oil droplets prepared from an ethereal extract of arbacia eggs, when exposed to a solution of the precipitate, decreased in diameter, in the course of two and a half hours, on the average, sixteen and seven tenths per cent. From this observation, Miss Woodward inferred that the fat was undergoing hydrolysis. Hence she called her precipitate, lipolysin.

Lipolysin is either not-agglutinin or it is denatured agglutinin. If the latter, then further search for an agglutinating substance in secretions from which lipolysin has been removed, should meet with little if any success. However a substance was isolated from just such remnants by saturation with ammonium sulfate, and the quantities so gotten appeared no smaller than those secured by the immediate saturation of the fresh exudates. The body precipitated in this fashion, when freed from salts, had no parthenogenetic properties. It did have marked powers as an agglutinant of sperm.

Whether Miss Woodward's further deductions and inferences will survive criticism matters not at all for the moment. Her results had the great virtue of preparing the ground for a different attack. In addition they made it possible to explain the effects of egg-secretions on spermatozoa and on eggs, in terms of two chemical individuals rather than in terms of one amboceptor with two side-chains.

This difference in the base from which interpretations must start, in the long run, may make small odds in the true inwardness of fertilization theory. I am not prepared to discuss the point. I am not even prepared to test the reasonableness of any particular answer pro or con for the conviction has overtaken me that I must practise birth-control in matters theoretical. But for one so minded, the one-body–two-body issue involves the whole matter of procedure. To me it is of great practical importance to determine, if possible, once for all, whether egg-secretions contain two substances or only one.

In his discussion of the question, Lillie[1] says: "This . . . does not explain why the sperm-agglutinating and the egg-activating properties of the egg-secretion always go together . . .; when the egg ceases to produce the sperm-agglutinating substance, it has lost its capacity to be activated. These two properties of the egg-secretion hang together normally; their separation under the conditions of chemical analysis may possibly denote a splitting of a single substance of the normal egg."

Unfortunately it would take us too far afield to discuss the essential question fully from the chemical side. Suffice it to say that during the last three years I have found other methods of isolation and in every case the agglutinating material and the egg-activating material are recovered as separate fractions. The amboceptor apparently has some pronounced physical weakness which causes it to break apart always at a point between the activating group and the agglutinating group. In fact this cleavage takes place so readily whenever one of the two groups combines with a precipitating agent that I have come to doubt whether the amboceptor can possibly hold together when its spermophile side-chain unites with the sperm-receptors.

However this may be, there is one bit of evidence which seems to me unassailable. Lillie proved that egg-secretions which have passed through Berkefeld filters do not cause the agglutinating reaction. The agglutinating material in this case, as Miss Sampson found last summer, and as I subsequently substantiated, can be recovered by washing the filter cone in sea-water. Whatever views one may hold regarding the physical-chemistry of

[1] "Problems of Fertilization," University of Chicago Press, p. 240.

filtration, that portion of the agglutinin which is recoverable in this manner is hardly the product of chemical breakdown suffered by the amboceptor molecule. For the ultra-conservative I may add that owing to the construction of the filter, a remnant of the secretion always fails to pass through. This remnant shows a higher agglutinating power than the original secretion. Moreover lest someone be led to suspect confusion, lipolysin can be isolated from the filtrates of such mechanical separations. Its presence in these is incidentally also revealed by activating effects on spermatozoa, a property the lipolysin shares with many other substances including preparations of pancreatic lipase.

III.

With our minds cleared on the one-body–two-body issue, we can ask and attempt to answer other specific questions. Among these we must include some further inquiries concerning the lipolysin. Is this substance really a lipolytic ferment? The evidence submitted so far is not conclusive because a decrease in the volume of oil drops might be the expression of changes in surface tension. But even if such shrinkage could be shown to result exclusively from hydrolysis, the question would still

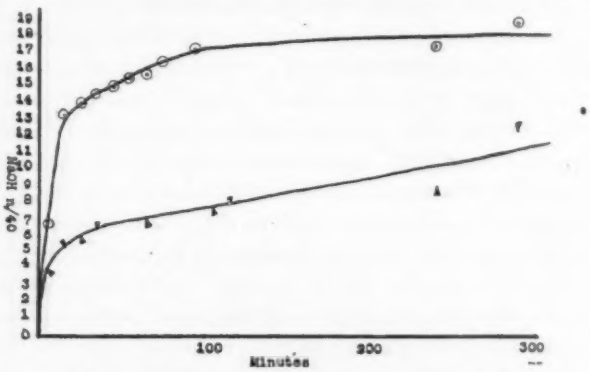

FIG. 1. Abscissa = minutes; ordinate = drops (1/100 c.c.) n/40 NaOH necessary to maintain system at PH 7. Both curves are the result of experimental averages and are slightly conventionalized. The lower one with experimental points at the apices of the triangles, represents the hydrolysis of ethyl butyrate in sea-water; the upper curve with experimental points in circles, represents the hydrolysis of ethyl butyrate in arbacia egg-secretion.

remain unsettled. An enzymatic effect is not demonstrated by hydrolysis, but by acceleration in the rate of hydrolysis.

Here again I cannot describe in detail the experiments which I have carried out during the last two years. However I am able to state very positively that the hydrolysis of neutral fats such as olive oil and whale oil as well as the cleavage of lower esters, such as ethyl butyrate are in fact accelerated not only by isolated lipolysin, but also by freshly prepared egg-secretions. The evidence, based on arbacia secretion, is given in the curves of Fig. 1. According to this lipolysin is correctly named for it is in fact a lipolytic ferment.

IV.

Can we come to similarly close quarters with the agglutinin? So far we have referred chiefly to the observable agglutination reaction. This, as Lillie found, is accompanied by other visible details. The agglutination of the spermatozoa is head-on. In *Nereis*, where the spermatozoa are larger than among the echinoderms, Lillie has described an actual swelling of the sperm-heads associated with a loss of normal refrangibility. He has suggested an increase of stickiness as the mechanism of agglutination.

The adhesiveness of cells unquestionably depends on surface properties and the effects of egg-exudates in general appear to be favorable to the view that agglutination is related to an incomplete superficial cytolysis of some sort. Aside from Lillie's evidence, which is susceptible to this interpretation, I have described a temporary agglutination of *Arenicola* larvæ accompanied by an outflow of pigment. Furthermore, isolated agglutinin has a marked effect on the surface of the egg. This quickly develops a clear zone immediately under the membrane—a result not only suggestive of membrane elevation but at the proper stage, quantitatively, perhaps the essence of this much debated process.

Richards and Miss Woodward, in their experiments with radiated egg-secretion, found evidence which suggests that very possibly the agglutinin also, is an enzyme. At any rate, radiation has opposite effects. If the secretion is radiated for two

minutes, its efficiency as a sperm-agglutinant is increased, but it is decreased if the radiation is extended to fifteen minutes. Moreover, regardless of radiation, the efficiency of the agglutinin, like that of pepsin, varies with the square root of the concentration. The presence of carbon and nitrogen may be mentioned incidentally. However, if the agglutinin is an enzyme, it is not yet possible to suggest its taxonomic position among organic catalysts. Before this can be done, we must find the process which the agglutinin catalyzes.

V.

The last question I shall discuss is the relation of lipolysin and agglutinin to the specificities of fertilization.

We know that specificity is not absolute; it is often possible to fertilize the eggs of one species with the sperm of another, yet species-true spermatozoa always fertilize a much larger percentage of eggs.

The discovery that chemical entities traceable to the eggs themselves intervene in fertilization—however obscure such intervention may appear—has exposed the problem of specificity from a new angle. Lillie has discussed the matter very thoroughly in his book.

For me, the questions involved take the following form: Is it possible to substitute the lipolysin of one species for that of another? Is it possible to do the same thing with the agglutinins? With regard to the lipolysins, the following experiments may be cited: secretion was removed from the eggs of *Echinarachnius* until their fertility had been reduced to one half. Separate lots were then inseminated in the presence of arbacia, asterias, oyster, and species-true lipolysin—all in a concentration of ten milligrams to five c.c. of sea-water.

The results are given in Table I. in which are listed, in separate columns, the percentages of inactive eggs, those of the anomalous cleavages, and those of divisions indistinguishable from the normal. Apparently, it is not essential that the lipolysin be species-true. More dramatic than this, of course, is Miss Woodward's recently announced discovery that arbacia lipolysin is an excellent parthenogenetic agent for *Nereis* eggs.

TABLE I.

	Per Cent. Inactive Eggs.	Per Cent. Active Eggs.	
		Anomalous.	Normal.
Control	46	54	0
Arbacia lipolysin	41	57	2
Asterias lipolysin	28	66	6
Oyster lipolysin	26	62	12
Echinarachnius lipolysin	32	60	8

For the agglutinins, hybridization offers a good approach.

Just ('19) has made successful crosses between *Arbacia* and *Echinarachnius*. If now the agglutinins of these forms play a specific rôle in fertilization, one might expect that this could be rendered apparent in hybridization experiments. For this reason I treated the sex cells prior to insemination with species-true secretion and compared the results with crosses in which this treatment was not given. The experiments condensed in Table II. speak for themselves.

TABLE II.

Eggs.	Treated with Egg-Secretion.	Sperm.	Treated with Egg-Secretion.	Per Cent. Cleavage.
Arbacia	none	*Arbacia*	none	100
Arbacia	none	*Echinarachnius*	none	1
Arbacia	none	*Echinarachnius*	*Echinarachnius*	13
Arbacia	*Echinarachnius*	*Echinarachnius*	none	9
Echinarachnius	none	*Echinarachnius*	none	100
Echinarachnius	none	*Arbacia*	none	5
Echinarachnius	none	*Arbacia*	*Arbacia*	40
Echinarachnius	*Arbacia*	*Arbacia*	none	22

In these experiments I used none of the expedients so often employed to break down the incompatibilities that limit success in hybridization. Neither the eggs nor the spermatozoa were allowed to grow stale; no alkali was used; there were no repeated inseminations, nor were the spermatozoa added to the eggs in greater quantities than usual. With the sole exception of the treatment whose efficacy I wished to test, I followed strictly all the procedures of ordinary species-true insemination.

I think it must be admitted, not only that there is an effect, but that this effect applies primarily to the spermatozoa. Since

now the lipolysins are apparently non-specific, I conclude, provisionally, that the agglutinating reaction involves specific elements in the sense that species-true agglutinin may have effects quantitatively and perhaps even qualitatively different from those of the heterologous agglutinins.

Whatever conclusions we may yet reach regarding the rôle of egg-secretions, we may legitimately at this time lay claim to success in certain directions and to some extent to forecast the future. Egg-secretions are not an isolated phenomenon; they have something vital to do with the initiation of development. Finally, the period of stumbling in the utter darkness is over, for our worst methods have reached some degree of reliability and some of them have yielded definite substances whose reaction-capacities mark off the zone within which we can hope to find understanding.

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HYDROGEN-ION CONCENTRATION OF PROTOZOAN CULTURES.

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The chemical changes taking place in ordinary hay infusions used for protozoan cultures have been described by various authors, among whom may be mentioned Peters[1] and Fine.[2] The points of interest discussed by these two authors are the changes occurring in the titratable acidity and alkalinity of such infusions. Changes in titratable acidity and alkalinity, however, as is well known, do not give a correct expression of the changes in concentration of hydrogen ions. Inasmuch as the hydrogen-ion concentration of the medium with which organisms are in contact is of great physiological importance, it was planned to study this factor throughout the life of series of protozoan cultures, and to see in how far such results agreed with those obtained by titration methods.

In the present study, therefore, determinations were made of both titratable acidity and alkalinity, as well as of hydrogen-ion concentration of series of cultures prepared in various ways.

The titratable acidity was obtained by titrating 5 c.c. of culture with 0.01 *N* NAOH, using phenolphthalein as indicator. The titratable alkalinity was obtained by titrating 5 c.c. of culture with 0.01 *N* H_2SO_4, using bromphenol blue as indicator. Hydrogen-ion determinations were made by colorimetric methods, using phosphate and acetate mixtures as standards, and phenol-sulfonephthalein, brom-cresol purple and methyl red as indicators. Series of cultures were prepared in various ways and determinations carried out daily. In the case of hay infusion cultures with hay, the same amount of hay (approx. 25 gms.) was used and treated in the manner indicated in explanation of figures. The soil in soil cultures was obtained from the greenhouse and was

[1] Peters, A. W., 1907, *Amer. Jour. Physiol.*, 17 and 18.

[2] Fine, M. S., 1912, *Jour. Exp. Zoöl.*, 12, p. 265.

that used by the gardener in ordinary greenhouse work. Total volume of cultures varied from 2 to 5 liters, depending on size of container used. When cultures were inoculated with organisms samples were taken from several cultures so that as many representative protozoan forms as possible were obtained. All cultures were kept under the usual laboratory conditions, in the same room, with the temperature as nearly constant as possible.

The data obtained in such a study are most readily presented by curves, since it is relative and not absolute changes in which we are interested.

Peters[1] and Fine[2] found that the top and bottom of cultures varied in titratable acidity—at first, a high titratable acidity at the bottom, and later, as diffusion proceeded, a more or less uniform concentration was obtained and titratable acidity differences tended to disappear. A similar condition has been found by the author in titrations, but the differences in hydrogen-ion concentration are never great and, when occurring, last only for a very short time in the early life of the culture. In the following results, however, average conditions only will be given.

Figure 1 shows graphically the changes in Ph in a typical soil culture. These cultures, during the first few days, become slightly acid, followed by a less acid condition, and, finally, remain rather alkaline throughout the remainder of the experiment. Observation showed that organisms were most abundant during the early life of the culture, when the change from a slightly acid to a slightly alkaline reaction was taking place.

Figure 1 also shows the changes in Ph in hay infusion cultures prepared in slightly different ways. From these results it is quite evident that the presence of hay, either boiled or unboiled, tends to make a decided difference in the hydrogen-ion concentration of the culture. No attempt was made to keep the cultures sterile, and since it has been pointed out repeatedly that the bacteria are to a great extent, if not entirely, responsible for the acid production, these changes in Ph between cultures with and without hay are in all probability due to amounts of available food for bacterial organisms, as well as to the acid-yielding con-

[1] Peters, A. W., 1907, *Amer. Jour. Physiol.*, 17 and 18.

[2] Fine, M. S., 1912, *Jour. Exp. Zoöl.*, 12, p. 265.

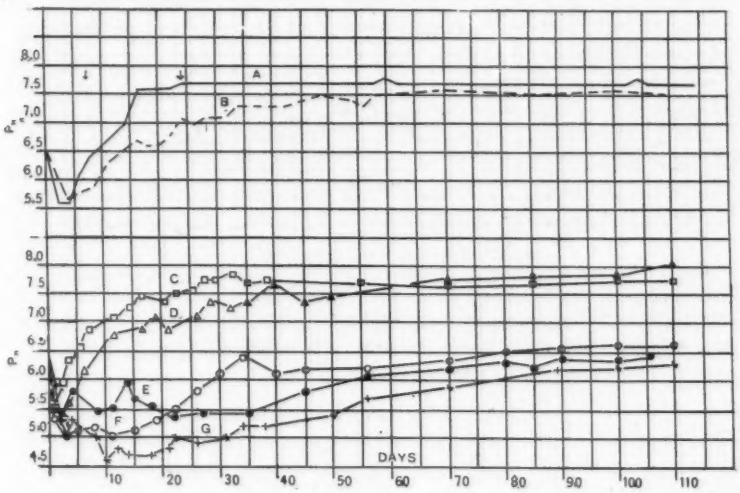

FIG. 1. *Top*—Curves show the Ph of soil and ordinary hay infusion cultures for a period of over 3 months.

A = soil culture, prepared with soil, hay infusion, tap water and inoculated with organisms.

B = culture prepared with hay infusion, tap water and inoculated with organisms.

Space between arrows indicates time when organisms appeared to be most abundant.

Bottom—Curves show the Ph of cultures with and without hay and also the Ph of inoculated and non-inoculated cultures.

C = culture prepared with hay infusion, tap water and inoculated with organisms.

D = culture prepared with filtered hay infusion and tap water and not inoculated.

E = culture prepared with hay, filtered hay infusion and tap water and not inoculated.

F = culture prepared with boiled hay, hay infusion, tap water and inoculated with organisms.

G = culture prepared with boiled hay, filtered hay infusion and tap water and not inoculated.

Volume of culture, 5 liters. Abscissas, time in days indicated. Ordinates represent Ph.

stituents of the hay. The differences in Ph between inoculated and non-inoculated cultures are probably due, either to a reduction of the bacteria by the protozoa, or possibly to various excretion products of the protozoa themselves.

It is also of interest to point out here that in the soil cultures there is a relatively short period of acidity followed by a longer

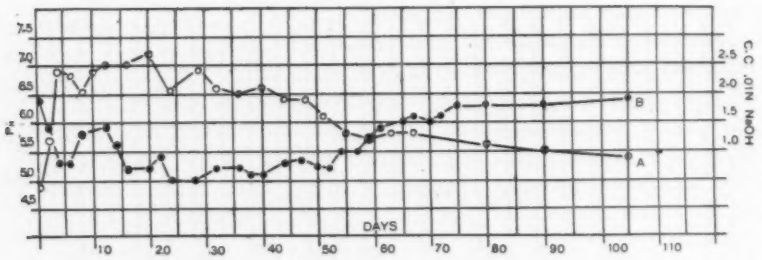

FIG. 2. Curves show the titratable acidity and Ph of an ordinary hay infusion culture, prepared with boiled hay, filtered hay infusion and tap water. *A* = titratable acidity. *B* = Ph. Abscissas time in days indicated. Ordinates at left represent Ph. Ordinates at right represent cubic centimeters 0.01 *N* NAOH required to neutralize 5 c.c. of culture.

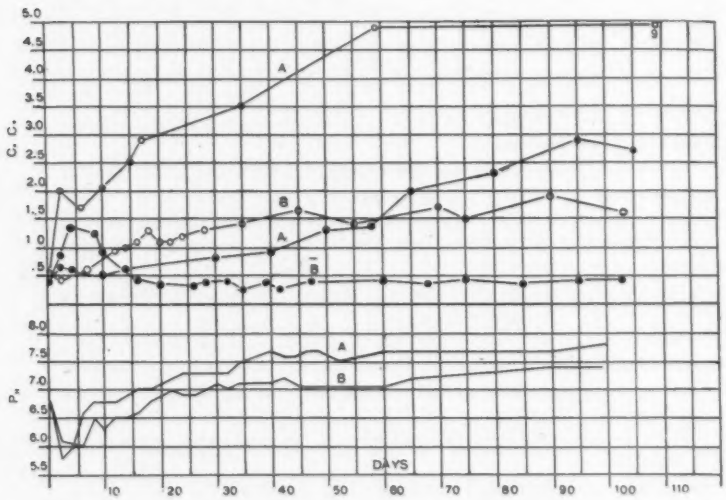

FIG. 3. Curves show the titratable acidity and alkalinity and the Ph of an ordinary hay infusion culture and of a hay infusion culture to which a piece of marble has been added. *A* = culture prepared with boiled hay, hay infusion, tap water, piece of marble and inoculated with organisms. *B* = culture prepared with hay infusion, tap water and inoculated with organisms. Volume of cultures, 5 liters.

Top—Curves show titratable acidity and alkalinity. O = alkalinity. ● = acidity. Abscissas, time in days indicated. Ordinates represent cubic centimeters 0.01 *N* NAOH and H_2SO_4 to neutralize 5 c.c. of culture.

Bottom—Curves show Ph of cultures. Abscissas, time in days indicated. Ordinates represent Ph.

one of alkalinity, while in hay cultures there is a period of acidity which lasts for a relatively long time in the life of the culture.

Figure 2 shows graphically the relation between the titratable acidity and hydrogen-ion concentration of a typical hay infusion culture. A somewhat more gradual and noticeable change is noted for titratable acidity than for hydrogen-ion concentration, and this seems to show that a marked change in titratable acidity is not necessarily accompanied by a correspondingly marked change in hydrogen-ion concentration.

Peters[1] and Fine[2] have shown that the acidity of hay infusions is due almost entirely to bacteria, to carbon dioxide, and acid-yielding hay constituents. This being the case, it is of interest to see how in an ordinary hay infusion a piece of marble will produce a fairly constant Ph.

Figure 3 shows graphically the changes in Ph and in titratable acidity and alkalinity in such a culture. These cultures gradually become alkaline in reaction, as indicated by Ph, but still have an appreciable titratable acidity. Cultures prepared in such a manner ought to be useful for keeping organisms in media of almost constant Ph.

The above results on titratable acidity and alkalinity are quite in agreement with those reported by Peters[1] and Fine,[2] and in addition show the relative changes in hydrogen-ion concentration during the life of hay infusion and soil cultures. The sequence of organisms and changes in hydrogen-ion concentration have not been particularly studied, but it is not improbable that some relation between the two might be shown to exist.

[1] Peters, A. W., 1907, *Amer. Jour. Physiol.*, 17 and 18.

[2] Fine, M. S., 1912, *Jour. Exp. Zoöl.*, 12, p. 265.

THE AXIAL GRADIENTS IN HYDROZOA.

C. M. CHILD,

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IV. AXIAL GRADATIONS IN RATE AND AMOUNT OF REDUCTION OF POTASSIUM PERMANGANATE IN VARIOUS HYDROIDS AND MEDUSÆ.

In an earlier paper (Child, '19*a*) attention was called to a method of rendering directly visible the physiological axial gradients in many of the simpler organisms and earlier developmental stages through the differences in rate and in total amount of reduction of potassium permanganate by the protoplasm of different regions and levels. Since the reduced permanganate (MnO_2 or other oxides) colors protoplasm brown to opaque black according to amount of deposit, differences in rate or amount of reduction in different regions appear as differences in depth of coloration or in degree of opacity.

In the paper referred to, the results of work with permanganate on various animal and plant species are briefly described and it is pointed out that the gradients in rate and amount of permanganate reduction correspond to the physiological gradients demonstrated by means of the susceptibility method and various other methods.

MATERIAL AND METHOD.

The present paper records observations made on the reduction gradients, as they may for convenience be called, of various species of hydroids and medusæ. The work was done at the Puget Sound Biological Station chiefly during the summers of 1918 and 1919 and I am indebted to the director for the privileges of the station. The data concerning the hydroids were obtained chiefly from four species, *Bougainvillea mertensi*, *Gonothyræa clarki*, *Obelia borealis* and *O. longissima:* but observations on rate of reduction were also made on several other campanularian species. Young hydroids were grown in the laboratory from the eggs of *Gonothyræa* and the hydromedusa *Phialidium gregarium*

and the physiological gradients have been followed through all developmental stages by various methods, but consideration of the embryological data is postponed to a later paper. Among the hydromedusæ four species, *Phialidium gregarium*, *Mitrocoma discoides*, *Æquorea cærulescens* and *Sarsia rosaria* constituted the chief material for the work with permanganate, but observations were made as opportunity offered on several other forms, *Stomatoca*, *Obelia* and several undetermined species.

The experimental procedure consists merely in immersing the species to be examined in a solution of $KMnO_4$ in sea water: For observations on the color gradients resulting from differences in rate of reduction concentrations ranging from $m/1000$ $KMnO_4$ down to $m/10{,}000$ or even less have been used in most cases. Higher concentrations than $m/1000$ may of course be used, but in such concentrations the color of the solution is so deep that the animals must be removed to water for observation. Moreover, the differences in rate of reduction and staining are more distinct in the lower than in the higher concentrations because the latter kill all parts almost at once in spite of the differences in physiological condition. Differences and gradients in total amount of reduction are determined by permitting the reduction to proceed to completion in excess of permanganate. Under these conditions most organisms become opaque black and no differences in depth of color can be seen, but in certain small organisms, embryonic stages, or regions of body, in which the thickness of protoplasm or cell mass is not too great, the color gradient resulting from differences in amount of MnO_2 deposited is directly visible. Many other forms which are opaque black in water may be made more or less translucent by hardening and gradual dehydration in alcohol and clearing in an oil, and if desired mounting in balsam. The axial gradients in blastulæ and other early embryonic stages of various species, as well as in the adults of various small forms, can be seen with great distinctness in such preparations. Larger organisms or regions which reduce relatively large amounts of permanganate may remain opaque even after clearing. In fact, the method is chiefly of value for small, more or less translucent organisms, but for these it gives very definite, uniform and beautiful results.

I have made no attempt to determine at all exactly the length of time necessary for completion of the reaction in given concentrations but have merely made certain that length of time was sufficient for the purpose. Usually preparations of this sort remained 24–48 hours in a solution of *m*/1000 or higher. My observations indicate, however, that in small organisms, such as hydroids, and in embryonic stages the reaction is in most cases complete in much shorter time, *e.g.*, 2–4 hours in *m*/1000.

All concentrations of $KMnO_4$ high enough to give any appreciable deposit of the oxide on or in the protoplasm are highly toxic. Even in *m*/20,000 ciliary activity ceases within a short time, ranging from a few seconds to about one minute in forms examined and traces of the color begin to appear on the external surfaces within two or three minutes. Except in those concentrations in which cytolysis and disintegration take place it is impossible to determine exactly when death occurs. Undoubtedly it occurs before reduction and staining proceed very far, for alteration in aggregate condition, apparently a coagulation, can often be observed in the cells in early stages of coloration.

Certain precautions necessary in the use of the permanganate may be noted. Certain low concentrations produce cytolysis and disintegration in many, perhaps in all, organisms. In such cases susceptibility gradients corresponding to those observed with other agents and to the reduction gradients appear. In concentrations producing disintegration the disintegrated mass may show more or less distinct gradients in rate or amount of reduction, provided it holds together, but commonly cytolysis is followed by loss of continuity and by distribution of the cell substance through the water. In higher concentrations the reduction gradients appear without disintegration.

In order to avoid irregularities in staining it is necessary to agitate the solution frequently or to move the organisms about in it. Reduction of the permanganate at the surface of the organism often occurs much more rapidly than its diffusion, and a zone of low concentration of permanganate may appear about the organism, particularly about its more rapidly reducing regions. Parts in contact with the glass or lying near other

reducing parts soon show a retardation in staining in consequence of decrease in concentration of permanganate in the region about them. For these reasons the volume of solution used should be large as compared with the volume of protoplasm and uniformity of concentration at all points of the protoplasmic surfaces should be maintained by continuous or frequent agitation.

One difficulty exists in the technique of permanent preparation of permanganate material either for whole mounts or for sections. All clearing agents used thus far remove more or less rapidly the black or brown deposit in the protoplasm, the MnO_2 being apparently soluble in or reacting in some way with the oils. This disappearance of color also occurs in Canada balsam. In the samples of clove oil used the disappearance of the color is rapid, *e.g.*, hydranths may fade from opaque black to light uniform yellow in two or three days. In cedar oil the fading occurs much more slowly and may be a matter of weeks. In the course of a study of permanganate preparations of larval stages of echinoderms Mr. A. E. Galigher has found that with rapid preparation sections could be obtained without appreciable loss of color, but that in balsam the color gradually disappeared. Small organisms, larval stages, etc., can be made sufficiently translucent by dehydration and clearing so that the differences in total amount of reduction are clearly visible and persist at least for a day or two, or with some clearing agents much longer.

It is scarcely necessary to point out that differences in thickness of the layer of protoplasm through which the light is transmitted may appear as differences in depth of staining. A hydroid stem of larger diameter, for example, may appear to be more deeply colored than a stem of smaller diameter and in such cases it is often quite impossible to determine whether the difference is real or apparent. Such difficulties arise chiefly in connection with preparations for total amount of reduction rather than with those for rate of reduction, for in the latter reduction and coloration occur first on the surface and progress inward, and the differences in rate of staining can usually be seen in early stages on the external surface of the protoplasm quite independently of its thickness.

Thus far an exact quantitative record of the differences in rate of staining has not been attempted because of the difficulties involved. It is difficult to determine exactly the moment when the staining begins in a certain region and although the regional differences in depth of color are very marked, even in forms as small as the hydroid planula or sea urchin blastula and gastrula, any adequate measure of this difference is not readily obtained. Photomicrographs can undoubtedly be made of stages of staining or of the cleared preparations, but in any case are little better than figures, and graphic methods are much less readily applied than in case of disintegration experiments (Child, '15, Cnaps. III.–VII.). In the present paper description is supplemented by a few diagrammatic figures in which the regional differences are indicated at some stage of coloration by degrees of shading.

Reduction Gradients in Hydroid Colonies.

Before describing the details it may be said that in general both the rate and, so far as it could be determined, the total amount of reduction, decrease from the axial regions basipetally. This holds not only for the single hydranths, hydranth buds, growing tips, medusa buds and stolons, but for the colony as a whole. The differences at different levels are least in the stems. The perisarc is of no very great significance as an obstacle to the passage of permanganate to the tissues within it. In the more apical regions parts inclosed in perisarc begin to stain almost or quite as quickly as naked parts, and even the thicker perisarc of the more basal regions of the stem retards the staining only slightly. The thickness of the perisarc, therefore, is certainly not responsible for the differences in rate of staining of different levels. The deposition of the oxide begins on the external surface of the protoplasm and a distinct regional gradient in staining is in many cases visible on the surface before any trace of reduction appears below the surface.

The Hydranth Gradients.—Reduction and coloration occur first at the tips of the tentacles and within a few moments each tentacle shows a distinct color gradient ranging from brown at the tip to light yellow in the basal region. Color begins to appear at the tip of the manubrium shortly after it appears on the

tentacles, never, so far as my observations go, at the same time, and progresses basipetally on the manubrium, the basal portions of the hydranth body being the last to show color. The color appears first on the external surface and it is not infrequently possible to see the formation of precipitate on the external surface of the cells. Such precipitate must result from reduction of permanganate on the external surface of the protoplasm and the reduction and coloration progress from the surface inward. The permanganate does not penetrate the living cell and kill after it attains a certain concentration in the interior but it reacts with the protoplasm and undoubtedly kills it as it comes into contact with it.

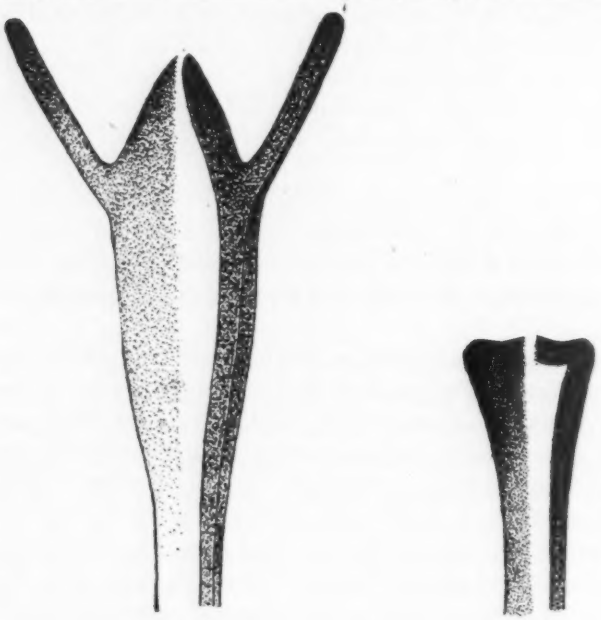
The color deepens rapidly and within fifteen minutes to an hour in concentrations ranging from $m/2000$ to $m/7500$ the regions where coloration first appeared are deep brown or opaque black and from these a color gradation to yellow in the basal regions appears.

After two to three hours the whole hydranth is usually opaque black in water. Teasing shows that at this time reduction has occurred throughout the ectoderm and that the entoderm is more or less deeply colored, and sometimes gradual differences in rate of entodermal staining can be seen, but the existence of entodermal gradients can be determined with certainty only in dehydrated and cleared material. After reduction is completed all hydranths are opaque black in transmitted light, and even after dehydration and clearing the bodies of the larger hydranths are opaque throughout, but the gradient is distinct in all tentacles and in at least the smaller hydranths.

The diagrammatic Fig. 1 indicates the color gradients in the tubularian *Bougainvillea*. The lighter side of the figure shows the gradient in rate of staining in single tentacle and the hydranth body, as seen from the surface after fifteen minutes to one hour in $KMnO_4$ according to concentration. The right side of the figure represents an optical section of tentacle and body wall after complete reduction, dehydration and clearing. The shading is intended to give some idea of the differences in depth of staining at different levels. After total reduction and clearing all parts

are very deeply colored, in fact large hydranths are often still completely opaque. The gradients are similar in the campanularian hydranth.

The Gradient in Hydranth Buds.—In *Bougainvillea* the buds show very distinct apico-basal gradients in rate of reduction from early stages on, and in material dehydrated and cleared after

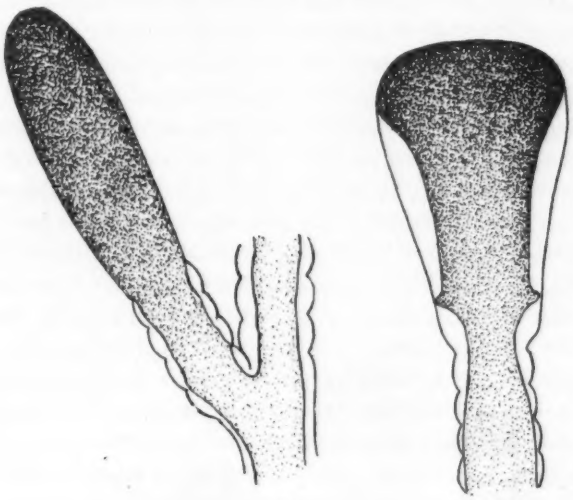


1 *2*

complete reduction the basipetal gradient appears in both ectoderm and entoderm. Figure 2 shows a somewhat advanced bud, the left side in surface view indicating the gradient in rate of reduction, the right side in optical section indicating the gradient in amount of reduction in cleared material.

In *Obelia*, *Gonothyræa* and other campanularians the axis of the colony is, as I have pointed out, sympodial and the bud which originated as a lateral bud below the terminal hydranth or the terminal bud undergoes considerable growth in length as a continuation of the main axis of the colony and may even give rise

to a new bud or branch before a hydranth develops at its tip (see Child, '19*b*, Fig. 10). These terminal regions of the campanularian colony and of its branches are regions of relatively rapid growth, each one representing temporarily a growing tip, and reduce $KMnO_4$ rapidly and in large amount. In spite of the fact that it is covered with thin perisarc, the coenosarc at the apical end of such a growing tip begins to stain earlier than any other part of the colony except the tentacles and in some cases the hypostome regions of hydranths. These growing tips and the hydranth buds which develop at their apical ends always show distinct basipetal gradients in rate of reduction, as indicated in Figs. 3 and 4. In preparations cleared after reduction is com-



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plete these parts in vigorous, rapidly growing colonies are usually stained so deeply that they remain opaque even after clearing, but these preparations show a much greater amount of reduction in these terminal regions than in the stems.

The Gradients in Stems.—All parts of the stem are of course enclosed in perisarc which increases in thickness basipetally. Reduction of permanganate does occur to a slight extent in the

perisarc; in the thin perisarc of the more apical regions the resulting color is scarcely appreciable, but the thicker perisarc of the more basal regions, which is normally yellowish or brownish in most forms, appears more deeply colored in the permanganate, probably because of its greater thickness. In no case observed, however, is reduction in the perisarc sufficient to obscure the progress of coloration in the cœnosarc.

The permanganate penetrates the perisarc rather rapidly. The apical regions enclosed in very thin perisarc begin to stain almost as soon as the naked parts. Penetration of the thicker perisarc of the more basal regions occurs somewhat more slowly, though even in these regions the perisarc is far from being impermeable. Because of the presence of the perisarc and its different thickness at different levels, difference in rate of reduction and staining of intact stems cannot be regarded as a safe criterion of the differences in physiological condition. This difficulty, however, is easily avoided by using pieces of stems from different levels with fresh transverse cut surfaces. Pieces from different levels may be placed side by side in permanganate and the rate of coloration of the exposed distal or proximal ends directly compared. In this way it is readily determined that a gradient in rate of reaction is present in the stems. This gradient is much less steep than that of hydranths and growing tips, for reduction occurs relatively slowly in all stem regions, but with differences in level of 10–15 mm. or more it is appreciable, and between stem levels near the apical and those near the basal end of colonies several centimeters in length the difference is marked. In such cases the more apical level usually becomes distinctly yellow or even brownish before the more basal level is appreciably colored.

In preparations of whole colonies or of the longer branches, cleared after completion of reduction, the stems may remain opaque throughout if the colony is well fed and the cœnosarc thick, but in most cases a distinct basipetal decrease in opacity appears. In order to make certain that such differences are not merely apparent and due to differences in transverse diameter of the perisarc, it is desirable to compare regions from the

different levels in which the diameter of the cœnosarc is approximately the same. Repeated observations of this sort leave no doubt that real axial differences in the total amount, as well as in the rate of reduction exist in stems and branches.

The Stolon Gradient.—Many hydroid species give rise under slightly depressing conditions to stolon outgrowths, not merely from basal or even from apical cut ends, but by transformation into stolons of hydranth buds, in campanularians, of growing tips or even apical ends or stems after degeneration of hydranths. Experimental data concerning such transformations will be presented in another paper. The stolon is readily distinguishable from the stem by indefinite direction of growth, by attachment to the substratum or other solid objects and in the campanularians by absence of the annulations characteristic of stems. The stolon, like other parts of the colony, shows a gradient both in rate and amount of reduction, decreasing from the tip. This gradient is less steep than that of the hydranth or the growing tip, but steeper than that of stems. Reduction of permanganate in the growing stolon is more rapid than in the stem, but less rapid than in hydranth buds and growing tips.

The Gradient of the Colony as a Whole.—The reduction gradient appears, not merely in the individual zooids and parts of the hydroid colony but there is a general gradient characteristic of the whole colony. The gradient of stem and branches already described above is of course a part of this general gradient, but this gradient also appears, like the susceptibility gradient (Child, '19*b*) in the differences between the hydranths and growing tips of different regions.

The *Bougainvillea* colony is monopodial like *Pennaria* (Child, '19*b*) and the primary hydranth of the colony or branch remains permanently the apical hydranth. Budding occurs subapically at a certain distance below the apical hydranth, and the new axes developing from the buds become lateral branches of the colony or branch each bearing its primary apical hydranth. In any complex colonial axis, the lateral branches grow less rapidly than the main axis and the physiological relations between the different axes are apparently similar to those existing in

plants of similar growth-form, *i.e.*, the chief apical region inhibits in some way and to some extent the lateral branches. It has been shown (Child, '19*b*) that these complex axes possess a general susceptibility gradient, the apical hydranth of the main axis being in general more susceptible than the apical hydranths of lateral branches and the apical hydranths of the more apical lateral branches, more susceptible than those of more basal branches. In *Bougainvillea* this general gradient is less distinct than in some of the campanularians with more definite growth-form.

In *Obelia* and *Gonothyræa* a similar general gradient appears in the whole colony and in the complex axial systems represented by its branches. In these forms, however, the growth form is sympodial (Child, '19*b*) and instead of a permanent apical hydranth each growing tip and the hydranth bud developing from it is temporarily apical, but is displaced by the next bud. The general colony gradient appears in the rate of reduction of the different growing tips and hydranth buds of an axial complex, the most apical showing in general the most rapid reduction.

In vigorous, rapidly growing colonies of *Obelia borealis* 5 cm. or more in length, taken from piles and floats, the general gradient is usually clearly visible to the naked eye by reflected light after reduction is completed or has progressed so far that the tissues are opaque in water. On removal to water and washing the depth of brown color of the tissues of such a colony, as seen by reflected light, decreases in general from apex to base in the colony as a whole and in each axial complex. The most apical growing tips and buds are very deep brown, almost black, the growing tips and buds of more basal regions distinctly lighter in color and even in the developed hydranths similar differences appear to some extent. The color gradient in the stem also appears in spite of the fact that the thicker perisarc of the more basal regions is more deeply stained by permanganate than that of more apical regions.

It is perhaps necessary to emphasize the point that these general gradients are most distinct in vigorous, growing colonies. The observations recorded above were made on colonies which

were known to have developed within three to four weeks preceding the observations and in which medusa buds or gonozooids had begun to appear only in the basal regions. I have pointed out elsewhere (Child, '19*b*) that medusa-bud development normally begins in colonies which are relatively old physiologically and in the most basal regions of the colony, *i.e.*, in the region of least susceptibility, and as the data of the present paper show, of lowest rate of reduction of permanganate. Later, as the colony grows progressively older, medusa-buds or gonozooids appear farther apically, until finally they may develop even from the apical regions.

The old colonies with medusa-buds or gonozooids in or near the apical regions may show little or no indication of a general colony gradient. Often in such cases the original hydranths of the most basal levels have disappeared, and in some cases new young hydranths may be developing. Colonies in this stage often show a higher susceptibility and higher rate of reduction in the newly developed, physiologically younger hydranths or growing tips of the more basal regions than in those of the more apical regions, which represent the original generation. Later, when these also have been replaced by a new generation the general colony gradient may once more become like that of the young growing colony.

In short, the gradient is not a fixed unchangeable condition which can be demonstrated in any hydroid colony. It is a feature of the normal developmental and functional relations of parts, but may be altered, obliterated or even reversed by environmental or physiological conditions. The gradients in the single hydranths and growing tips are more persistent. So far as my observations go, their disappearance is followed by the disappearance through death in the case of more highly specialized, or resorption in the case of less high specialized parts. Similarly, in the colony as a whole the disappearance of the general gradient is associated with the disappearance, at least temporarily, of the original order and relation characteristic of the colony. Old hydranths or axial complexes may die or be resorbed and new ones may develop, but my observations indicate

that wherever new development of a hydranth or an orderly axial complex occurs the characteristic gradients are found to be present and after a period of resorption, redifferentiation and rejuvenescence following the stage of medusa-bud formation the general colony gradient may reappear simply as a physiological consequence of the order in which development, senescence, death or resorption, and new development occur in different parts of the colony. In other words, the new colony gradient in such cases is a physiological consequence of the existence of the original gradient. Of course, in some cases, environmental factors may play a part in altering the original relations and so in modifying the form and order of the colony.

It is of interest to note that Gast and Godlewski ('03) studying the form regulation of *Pennaria cavolinii* found a general colonial gradient in rate of regeneration of hydranths. After removal of the hydranths from a colony or axial complex, development of new hydranths decreased in rate basipetally in the colony as a whole and in each axial complex. Later degeneration of the regenerated hydranths began in the most basal regions and proceeded a greater or less distance apically, but the most apical hydranths persisted, grew and budded at the expense of these other more basal parts. Both of these gradients, the regeneration gradient and the degeneration gradient, indicate that the fundamental physiological activity of the protoplasm is greater apically and decreases basipetally in the colony as a whole and in each axial complex. In short, these regulatory phenomena constitute still another line of evidence for the existence of the physiological gradients.

And finally, I am permitted to state from unpublished work that Dr. A. W. Bellamy and Dr. L. H. Hyman, working independently, have found gradients in electric potential in hydroid colonies of various species. These electrical gradients correspond to the gradients in susceptibility, reduction of permanganate and rate of regulation. In vigorous, growing colonies the apical region of the colony is galvanometrically negative to all other levels, the apical region of each axial complex is negative to all other parts of that complex, and the apical regions of the

more apical branches are negative to the apical regions of the more basal branches.

The Gradient of the Medusa Bud.—In the naked medusa buds of *Bougainvillea* and other tubularians the gradients in rate and amount of reduction of $KMnO_4$ appear very distinctly. The free apical end of the bud always shows the highest rate and greatest amount of reduction, as indicated in Fig. 5, a young bud of *Bougainvillea*. The medusa buds of the campanularians constitute less favorable material because the crowding along the blastostyle and the presence of the gonotheca render it impossible to insure uniform concentration of permanganate over all parts of the surface. In general, however, it is perfectly clear that the apical regions of these buds reduce permanganate more rapidly and in greater amount than other levels.

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In *Gonothyræa* the rudimentary medusæ or gonophores in the later stages of development come to lie outside the gonotheca, though still attached to the blastostyle, and so are directly exposed to permanganate solutions. The rate of reduction is highest in the small tentacles of these gonophores and decreases basipetally over the outer surface of the body. The subumbrellar cavity of the gonophore is at first closed and never widely open to the exterior, so that it is not possible to determine with certainty the relative rate of reduction in this region, though it is probably much the same as in other medusæ. In the old gonophores after extrusion of the planulæ the reduction gradient may be almost or entirely absent.

Reduction Gradients in Free-living Hydromedusæ.

The simple experiment of placing medusæ in a permanganate solution and agitating sufficiently to provide for uniform distribution of the permanganate was performed repeatedly during three summers and always with essentially the same result. In all forms examined reduction occurs most rapidly in the marginal tentacles and the oral lobes or tentacles, when such are present. Each tentacle or lobe shows a gradient in rate of reduction,

decreasing basipetally. Elongated manubria show similar gradients in rate of reduction. Manubrium and margin are regions of more rapid reduction than other subumbrellar regions, but the subumbrellar ectoderm in general reduces more rapidly than the exumbrellar ectoderm. Dehydration and clearing is not necessary in the medusa species used because the cellular tissues are mostly in such thin layers that they do not become opaque black. Nevertheless, the final difference in depth of color between exumbrella and subumbrella cannot be regarded as a fair criterion of differences in total amount of reduction because the thickness of cellular tissue is much greater in the subumbrella than in the exumbrella. On this account I am inclined to regard the differences in rate of reduction as more significant than the final differences in depth of color. The jelly reduces little permanganate and reduction is very slow.

Data on susceptibility of medusæ have not previously been recorded, but numerous experiments have been performed, chiefly on *Æquorea*, but in part on other species, with various concentrations of several agents, viz., KNC, *m*/100–*m*/500, HCl, *m*/200–*m*/800 and KOH, *m*/200–*m*/600. The susceptibility gradients in all these agents are essentially the same as the reduction gradients. A few experiments with neutral red and methylene blue and various other agents on the smaller forms gave similar results. It may be said then that here, as in the hydroids, the susceptibility gradients and the reduction gradients correspond.

It is of interest to note that the higher susceptibility and more rapid reduction of the subumbrellar as compared with the exumbrellar regions is in accord with McClendon's data on oxygen consumption in *Cassiopea*. He found that the subumbrella consumed much more oxygen per unit of weight than the exumbrella plus the greater part of the mesoglœa (McClendon, '17). Dr. Bellamy and Dr. Hyman have found differences in electric potential corresponding to the differences in susceptibility and rate of reduction, not only as regards subumbrella and exumbrella, but also as regards marginal and oral, as compared with other subumbrellar regions. The regions of highest galvano-

metric negativity correspond in all cases to the regions of highest susceptibility and rate of reduction.

Reduction after Killing.

The gradients in rate and amount of reduction of permanganate appear when the living animals are brought into permanganate, but when they are first killed by some other means and then brought into permanganate the gradients are either entirely absent, or only the merest traces of them remain. Various means of killing have been used in these experiments, among them $HgCl_2$, $HgCl_2$ with 5 per cent. acetic acid, HNO_3, HCl, strong alcohol, formalin, hot sea water. If the animals are brought into permanganate within a few moments after the killing agent is applied, with only a brief washing in sea water, if necessary to remove excess of the killing agent, slight gradients may still appear. So far as my observations go, however, this is not the case if the animals are subjected after killing to the usual treatment of histological material. If, for example, they are passed through the alcohols up to 70–80 per cent. and kept in this percentage for a day or two, or if they are killed and kept in formalin and then washed in water before being placed in permanganate, the rate of reduction in different parts is uniform and the only differences in depth of color, so far as could be determined, are the apparent differences resulting from differences in thickness of the layers through which the light passes. Moreover, in these killed animals the total amount of reduction is much less than when the living animals are brought into permanganate. Potassium permanganate, even in high dilutions, is highly toxic and when the living animals are placed in it they are of course killed by it, but it is evident from the facts cited that the differences in physiological condition in different regions play a part in determining the rate and amount of reduction. In the dead animals these differences are no longer present, although some factor or factors concerned in them may persist for a time after the killing agent is applied. Whatever the nature of the differences in reaction to permanganate between living and dead protoplasm, it is evident that the gradients in

rate and amount of reduction are associated with and dependent upon the living condition and disappear, either with, or soon after, death.

Conclusion.

Since $KMnO_4$ is a powerful oxidizing agent, the inference is justified, as I have pointed out elsewhere (Child, '19*a*), that the rate and amount of reduction of permanganate by living protoplasm is in some way and to some degree related to oxidative activity in the protoplasm. This inference is apparently supported by the marked decrease in reducing capacity of the protoplasm with death and the disappearance with, or soon after death, of the regional differences or gradients in rate and amount of reduction. Moreover, the reduction gradients are essentially the same as the susceptibility gradients and many different lines of evidence indicate that the latter are, at least to some extent, associated with and dependent upon differences in rate of oxidation (Child, '20). The results obtained with permanganate are then in complete agreement, as regards the existence of physiological gradients, with those obtained by other methods and constitute another line of evidence in support of the general conception.

The question of the rôle of permeability in relation to the reduction gradients must be raised. It has been repeatedly pointed out (Child and Hyman, '19, Child, '20) that gradients in permeability are in many, if not in all cases, characteristic features of the physiological gradients, but many facts show clearly enough that the fundamental metabolic activity of the protoplasm is also concerned in these gradients. We are forced to conclude, either that the gradients are not simply permeability gradients, or else that permeability and the fundamental metabolism of the protoplasm are very intimately associated and more or less interdependent. As regards the reduction gradients, it may seem at first glance that the differences in rate of reduction are merely the result of differences in permeability to permanganate. This is perhaps to some extent the case, but observation indicates that these differences appear first upon the external surface of the protoplasm. It is not necessary that the per-

manganate shall penetrate to the interior of the cell before the reaction begins. Moreover, the protoplasm in which the reaction occurs is undoubtedly killed very rapidly, as is indicated by the almost instantaneous cessation of ciliary and flagellar activity, even in very low concentrations of permanganate. It is certain that the permanganate does not penetrate the plasma membrane or any other limiting surface to any appreciable extent while that surface is living, but rather kills or begins killing as it comes into contact with the surfaces. My observations indicate, however, that the differences in rate of reduction disappear after death. If this is true, permeability in any physiological sense, *i.e.*, permeability of living protoplasmic limiting surfaces, cannot be the chief factor in determining the gradients in rate of reduction, for any differences in such permeability must disappear very soon on contact with the solution. If the particular limiting surfaces persist as limiting surfaces after the action of permanganate, and it is by no means certain to what extent they do persist, their permeability is no longer the physiological permeability of the living surface, but simply that of a dead surface. In short, after a given limiting surface, whether external or internal, has been killed by permanganate, the further passage of that surface by permanganate is not determined by its physiological permeability. If this argument is correct, the differences in rate of reduction of permanganate determined by differences in physiological permeability should be slight, since permanganate is highly toxic, but, as a matter of fact, the differences are very great when living animals are brought into the solutions and absent, or almost absent when dead animals are used.

Moreover, the deposition of MnO_2 or other oxides in the protoplasm is the result of a chemical reaction between the permanganate and protoplasmic constituents and therefore depends on other factors than the mere entrance of the agent. There is every reason to believe that the chemical and particularly the oxidative activity of the protoplasm is a factor in determining the velocity of the reaction, even though death occurs rapidly. Exactly what changes constitute death in permanganate, or for that matter in any other agent, it is impossible to say, but it is

evident that the beginning of death does not stop the reduction of permanganate, and even after death is complete the reaction occurs to some extent. The slight regional differences in rate of reduction which sometimes persist for a time after death is apparently complete may perhaps mean that certain death changes, *e.g.*, inactivation of oxidizing enzymes, stabilization of oxidizable substance, are not yet complete, but at most these differences are mere vestiges of the original differences.

And finally, the regional differences in the total amount of reduction cannot be due to differences in the permeability of limiting surfaces. In the light of all the facts concerning the physiological gradients, the only conclusion at present justifiable seems to be that they are associated with and dependent upon differences in the chemical, and particularly the oxidative activity of the protoplasm concerned, though it is, of course, granted that various factors are concerned in these chemical differences. The reduction gradients appear only when living protoplasm reacts with permanganate, or as mere vestiges in protoplasm very recently killed. The regions of greatest amount of reduction are the regions of greatest physiological activity, as indicated by growth, development, susceptibility, electro-negativity, and in those forms for which it has been determined, of respiratory activity. I believe we are justified in concluding that, when used with proper precaution and in connection with other methods, the rate and amount of reduction of potassium permanganate by organisms which are alive at the beginning of the reaction serves as an indicator of the fundamental physiological condition of the protoplasm. This conclusion holds, not only for hydroids, but for all other forms examined (Child, '19*a*).

Summary.

Hydroids and hydromedusæ placed in dilute solutions of potassium permanganate show characteristic axial gradients in rate and total amount of reduction of permanganate, as indicated by the coloration of the protoplasm by MnO_2 or other products of the reaction. These gradients correspond with the physiological gradients indicated by other methods and by the development, growth-form and functional activities of the organisms.

The gradients are present, not only in each zooid of the hydroid colony and in each tentacle of the hydranth, but in each axial complex, *e.g.*, a compound branch, and in the colony as a whole, provided it is vigorous and growing. In general the rate and amount of reduction decrease basipetally. In the medusæ reduction is most rapid and greatest in amount in the marginal and oral regions, less in other subumbrellar regions and least in the exumbrellar region. Animals killed by other means before reaction with permanganate show no gradient, or in some cases vestiges, if newly killed. The conclusion is drawn that differences in rate and amount of reduction of permanganate, as indicated by the coloration, may, with proper precautions, be used as an indicator of differences in fundamental physiological condition in different regions of an organism.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 4. THE PHOTIC SENSITIVITY OF OGILBIA.[1]

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Parker ('09) has commented upon the curious fact that although so primitive a chordate as ammocœtes exhibits a well-defined integumentary sensitivity toward light, marine fishes as a class seem devoid of this type of irritability, which is also absent in *Amphioxus* (Parker, '08; Crozier, '17). Recently Jordan ('17) has described the first recognized instance of photic sensitivity resident in the skin of a marine teleost, the hamlet (*Epinephelus striatus*). I wish now to record a second example of this type of sensitivity in a marine fish, *Ogilbia* (*Brosmophycis*) *verrillii* Garman.

The case of *Ogilbia* is of peculiar interest. The Brotulidæ, to which group *Ogilbia* belongs, are for the most part deep water fishes, but include several forms occurring in warm shallow situations on the Pacific and on the Atlantic shores of America. They seem to represent the ancestral type from which may be traced the evolution of the blind brotulids of the Cuban caves (Eigenmann, '09). The behavior of *Ogilbia*, concerning which very little has been known, should in consequence be a source of important information bearing upon the derivation of the related cave forms.

Ogilbia verrillii (Garman, '00) was first collected at Bermuda by Verrill. According to Eigenmann ('09, p. 187)—who, curi-

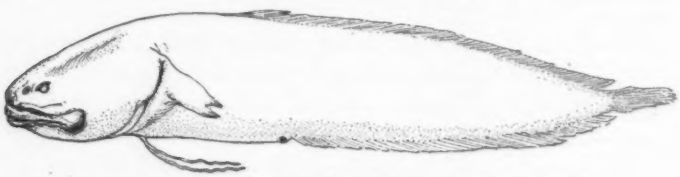

FIG. 1. *Ogilbia verrillii* Garm. (× 2½). Note the relatively minute eye.

[1] Contributions from the Bermuda Biological Station for Research, No. 130.

ously enough, in his review fails to mention the significant occurrence of *Ogilbia* at Bermuda—other species occur (*O. ventralis*) "in rocky pools about the Gulf of California and at La Paz," and (*Brosmophycis cayorum*) "on a shoal covered with algæ at Key West." My own specimens of *O. verrillii*, obtained singly and at scattered intervals, were secured from widely separated points about Bermuda—along the shore of Flatts Inlet, on the north side of Dyer Island, in Fairyland Creek, and in Tucker's Town Bay. In each case the habitat was at about low water level, either under a rock of some size or else in a dense mass of reddish and brown algæ. The length of these individuals was not over 4.5 cms. In color they were brownish yellow, speckled with minute red dots; there was little indication of counter-shading. They are not vigorous swimmers. When exposed to bright sunlight, by the sudden overturning of a rock slab, they seemed stupefied, and in several instances were easily secured by hand.

I wished to examine the behavior of these fishes toward light, and although but six specimens were available for experimentation, at different times from April, 1916, to December, 1917, the observations made seem adequate for certain purposes.

These fishes were intensely reactive to sunlight. If placed in an aquarium with one end illuminated from above, they remained almost stationary in the shaded portion, and if caused to swim toward the lighted area they turned back almost instantly when the anterior end came into the light. With direct sunlight, temperature 26° C., the reaction time averaged 1.3 seconds. Rheotactic response was also well marked, the animals heading in the usual way into a current. Taking advantage of this response, by appropriately directing a mild stream of water into a circular dish, it was possible to induce swimming movements without touching the fish.

Stimulated in this way, an *Ogilbia* would swim slowly up to the edge of a sharply defined sunbeam and remain stationary in the shade. Comparatively violent, undirected, swimming could be induced by suddenly illuminating the whole dish. Swimming movements ceased almost immediately when the light was

obstructed. *Ogilbia* is very strongly thigmotactic and in most cases settled in the angle between the bottom and the wall of an aquarium.

With two specimens it was possible to remove the eyes and to keep the animal alive for about 24 hours thereafter. The eyes are quite small (Fig. 1) and burning operations were unsuccessful, two fishes being killed in attempts to obliterate the eyes in this way. The eyeless fishes were also found to be sensitive to light, and to orient, with some precision, away from it. The reaction time to direct sunlight was, in the one case measured, about 3 seconds.

Even with normal specimens, it was possible to induce weak swimming by illuminating merely the hinder half of the body.

There seems consequently no reason to doubt the occurrence in *Ogilbia* of a true photic sensitivity of the skin—a result of special significance in view of the integumentary photic sensitivity found in *Amblyopsis* (Payne, '07), and of the fact that the blind Cuban brotulid *Lucifuga* (Eigenmann, '09, p. 199) seems probably to be photonegative (although the data are scanty). This type of irritability is rather characteristic of cave vertebrates, and its presence in *Ogilbia* is perhaps susceptible of a significant interpretation. In Eigenmann's view, the blind cave fishes *derive* from types originally photonegative, living in darkness under stones along "coral" reef shores, which survived a process of adaptive adjustment to fresh water and to cave environments conditioned by gradual elevation of the land.[2] It is frequently assumed that special modes of behavior, notably those dependent upon enhanced photic and tactile irritability, represent new developments determined in relation to blindness and to the conditions of cave environments. Hence it is important to recognize that even a manifestation so rare among marine fishes as skin sensitivity to light, is adequately exemplified in a teleost, not cave-inhabiting, but with eyes seemingly degenerate and on other grounds assigned as representative of the ancestral origin of typically adjusted cave forms. The state of pre-adaptation

[2] It may be mentioned that I have several times made search for fishes in cave pools at Bermuda, but have never encountered fishes in them, save where obvious communications led to outside water.

thus indicated is reflected also in connection with tactile irritability (Crozier, '18).

Summary.—Ogilbia verrillii, shore-living brotulid and close relative of blind brotulids of Cuban caves, has relatively small eyes and is decidedly photonegative in its behavior. Its skin is sensitive to light, a condition thus far recognized in but one other marine fish; this is taken to be of significance for the conception of preadaptation.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 5. THE PHOTOTROPISM OF LIMA.[1]

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The monomyarian lamellibranch *Lima* is said (Kafka, '14, p. 393) to be "photoptic," *i.e.*, reactive to light but not to shadows. The statement may be applied to the species of *Lima* found in some abundance at Bermuda, but there are certain additional features about the photic responses of this animal which merit a more extended notice.

Polimanti ('12) has distinguished three types of locomotion in *Lima hians:* (1) by the use of the foot as a lever; (2) by closure of the valves and retraction of the tentacles (Butler, '91); (3) by the expelling of water through the siphons. The first of these types may however have some complex aspects.

Lima was usually obtained near low water, around the edges of large stones, and among smaller stones piled together loosely, in bays where a certain amount of silt was being deposited; but was also encountered on the reefs, in crannies between corals, and sometimes among colonies of *Ecteinascidia*. It is decidedly photonegative in its behavior, although this activity may be lost after some days of laboratory captivity in open dishes. The edge of the mantle is beset with long tentacles, which are only very slightly adhesive toward wood or glass, but upon which carmine particles stick and accumulate readily. When touched, these tentacles react individually, by a vigorous and complicated retraction, but the reaction time is curiously long (about 1 minute, after a vigorous prod). They are reactive also to strong currents of sea water, and respond vigorously to the local application of a gentle stream of fresh water. At rest, the long white tentacles are widely extended (for a good figure, cf. Nutting, '19, p. 93). The tentacles are quite sensitive to weakly acid solutions; and, after the animal has been for a time in darkness,

[1] Contributions from the Bermuda Biological Station for Research, No. 131.

illumination seems to cause a variable number of them to curl and contract, but not sharply; they do not themselves appear locally sensitive to light. Stimulation of a tentacle by weak acetic acid in sea water caused the particular tentacle to retract instantly, and the animal to immediately swim violently away.

In swimming, *Lima* has open to it a variety of possibilities. It may progress while lying on either side, after the manner of *Pecten*, or it may swim with the medial plane in the vertical position, the animal being out of contact with the substratum save between "steps." The "steps" may in either case be made in one of several ways—either toward the gape of the shell, as with *Pecten*, in which event the mechanics of the swimming act is similar to that of *Pecten* (Vlès, '06; Bauer, '12); or else toward the hinge, by squirting water from between the valves. The mantle fold is relatively wider than in most Pectinidæ, and is very mobile; at rest, it beats with a rhythmic pulsation, doubtless functionally homologous with that known in some other lamellibranchs (Redfield, '17), but different in one regard: in *Anodonta* and in other clams the opposite mantle-flaps beat synchronously, whereas in *Lima* they unmistakably pulsate in opposite phase, alternately.

The *Pecten*-like mode of progression is the one usually adhered to, whether the animal be swimming vertically or on one side (and by actual count it was found that either side is used, indifferently). This is almost the only type of swimming induced by photic stimulation, the other mode of progression, in the direction of the hinge, being slower and made up of very short "steps."

When a previously darkened *Lima* is illuminated by horizontal sunlight, it turns the gape away from the light and swims, usually with the valves vertical, in a straight line away from the light. There is exhibited a definite and peculiar process of orientation. The long, mobile foot is thrust out between the valves, its free portion is bent sharply away from the light, and its distal end becomes adherent to the substratum. The foot then shortens longitudinally, so that, without moving its valves, the body is turned somewhat away from the light; several

"steps" of this sort, perhaps three in all as a rule, result in an almost perfect alignment of the median plane in the axis of the light beam; the *Lima* then swims, *Pecten*-wise, in a straight line away from the light. The swimming act is not usually prolonged, perhaps 50 cms. representing the maximum distance covered before temporary exhaustion intervenes, but the orientation process is of a definite, clean-cut character and was previously unknown, so far as I am aware, among bivalves. It is a point of some interest that in a photosensitive form such as *Lima*, where structural conditions give the possibility of phototropic behavior, we do in fact find such behavior diagrammatically exhibited.

The process of body-orientation is usually as just described; but if light be directed upon the gape of a vigorous *Lima* it sometimes gives a violent somersault, turning completely through 180° by some mechanism which I was not able to analyze; the subsequent swimming is in the usual manner.

I tried to find out if the foot, so active in the commoner orientation process, were itself photosensitive. After various trials with local illumination I came to the belief that it was, but complete proof of this I did not secure.

It is especially when the animal is weakened by handling or by laboratory conditions that swimming on the side is found. When in good condition a *Lima* can swim for at least 25 cms. through the water without touching substratum.

The photonegativity of *Lima*, involving so curious a method of orientation, is obviously important in maintaining these animals in crannies and darkened clefts, under stones and among coral heads.

Summary.—The bivalve *Lima* is photonegative, and in vigorous individuals the response to illumination involves a true and accurate orientation of the body, with gape away from the light, by means of the long adhesive foot, which is itself perhaps photosensitive; orientation is followed by *Pecten*-like swimming, the valves being vertical.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 6. RELATION OF LIGHT TO THE PIGMENTATION OF ASCIDIANS.[1]

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1. Experiments concerned with the re-formation of the test of the blue-black *Ascidia atra*, subsequent to its injury or partial removal, have demonstrated (Hecht, '18a, p. 236) that "the newly formed test material is pigmented in the usual way," even in the case of animals operated upon at night and maintained during regeneration in complete darkness. Since, "moreover, a new, pigmented test will form on the right face of the animal under the intact, opaque, old one, when the latter has been accidentally separated from the ectodermal surface which secretes it," Hecht concluded that the characteristic pigmentation is not due to the presence of light—in the sense, at least, that it cannot result from "a photic stimulus only." This conclusion is of interest, since it at first sight conflicts with some facts afforded by related species, and also with facts which may be made out from the distribution of *A. atra* itself in nature. Hecht's experimental result I can fully confirm, but inasmuch as pale, relatively unpigmented individuals are to be encountered in dark situations, the relation of the illumination to pigmentation seemed far from clear.

It might be assumed that if, in the normal course of development, pigmentation were simply a qualitative result of metabolic events, the quantitative course of transformations leading to pigment deposition in the test might be determined, once for all, by illumination early in the ascidian's life; if this were correct, experiments upon adult animals might be quite incompetent to decide whether or not light has anything to do with pigmentation—and effects substantially of this category are not unknown (Goldfarb, '10).

[1] Contributions from the Bermuda Biological Station for Research, No. 132.

2. Owing to the nature of its normal mode of occurrence, which is most frequently in bright, sunlit situations in shallow water, *A. atra* is rendered quite conspicuous by its dense blue-black coloration—so much so, in fact, that some observers (cf. Mortensen, '17) have not hesitated to suggest the operation of "warning" coloration in this connection. In view of this fact, and of the direct relation, in *Ascidia*, between pigmentation and blood metabolism (Hecht, '18a), the finding of pale individuals in dark situations required further analysis.

Simple ascidians having transparent or translucent bodies are commonly found to frequent dimly illuminated spots. Examples are given by *Ascidia curvata*, quite transparent and at Bermuda found almost always under fair-sized stone slabs, although taken by me also among masses of the alga *Valonia*, the dead cells of which are closely imitated by it (!); by *A. californica* (Ritter and Forsyth, '17), from the undersides of rocks; and by *Ciona intestinalis*, "especially given to clinging to the undersides of floats, buoys, and the like." The original assumption of a darkened habitat by these forms is probably due to the fact thar the ascidian larva, at first photopositive, becomes sooner or latet negatively heliotropic, before attaching; in different species this reversal of behavior with respect to light may be much delayed, or may even not appear at all. (In another place I shall discuss experiments concerned with the behavior of the tunicate tadpole.) It is of interest to note that the adult *Ciona* is quite reactive to light, in a sensory way, and also that in the laboratory these animals die rather quickly in diffuse light, although in dim light or darkness they are able to live for some days (Hecht, '18a); whereas *A. atra*, adult, is insensitive to photic excitation (Hecht, '18b; Day, '19).

Very pale examples of *A. atra*, however, were, as stated, found under stones in various places at Bermuda. Some of these were carefully gathered, and used in the following experiment:

"Aug. 1 to 7, 1918. Under stones on the shore of Trunk Island, Harrington Sound, 4 very pale *A. atra* secured; blue-black pigmentation was apparent only at the very tips of the siphons. These were kept in laboratory dishes lighted by a northwest window, some little direct sunlight falling upon the

animals late each afternoon. The tunicates were perceptibly blacker on the second day, and rather dense black by the seventh day."

The experiment was several times repeated. The individuals concerned were 1.2 to 2 cms. long.

From such results it might appear that in the case of *A. atra* light has indeed a direct effect in producing dark pigmentation, as it undoubtedly has in certain molluscs (cf. List's observations, 1902, on the superficial pigmentation of mussels; these observations I can confirm from experience with the Bermudan littoral *Modiolus*). But it is known that a number of tunicates change color under laboratory conditions (Caullery, '95; cf. also Holt, '14). Accordingly, I repeated the above experiment, with the difference that the animals taken from under stones were immediately placed in darkened collecting buckets, and in the laboratory maintained in aquaria in a dark room. By the sixth day, these individuals were as dark as the similar ones exposed in the laboratory to illumination. Hence it cannot be decided from such experiments whether or not light has any direct significance for pigment-deposition in the test of *A. atra*.

3. A possible explanation was sought in another direction. Every *A. atra* found on the under side of a stone slab was relatively small, none being more than 4 cms. long. Individuals of this length collected from reef rocks, wharves, buoys and floats, moreover, were comparatively much more robust. As an index of better growth, the thickness of the test on the right side of the body was measured in several of these. This thickness, in the normally situated ascidians, amounted to 2.0 ± mm.; whereas in those living under stones the thickness of the test was but 0.8 mm. Since it is fairly certain that the blue-black granular pigment of the *A. atra* test derives from metamorphosed vanadium-containing blood cells (Hecht, '18c), and is essentially an excretory product (Crozier, '16), as seems probable for some other ascidian pigments also (Pizon, '01), it is not unreasonable to suspect that malnutrition may be in these instances responsible for faulty pigmentation. Fuchs ('14) observed, in laboratory cultures, that the siphons of young Cionæ lengthen remarkably in

proportion to the available food material, and was able to correlate this finding with the forms of *Ciona* in different natural environments. *A. atra* from among piled stones was noticed (before Fuch's paper was known to me) to have siphons very short in comparison with those of the more robust specimens from open situations. The view is, therefore, permissible that relatively deficient metabolism is in this instance responsible for subnormal pigment deposition.

The experiments of Miss Johnson ('13) regarding the pigmentation of amphibian larvæ showed the kind of materials ingested, rather than the absolute amount of food, to be important for pigment formation. In *Ascidia* we are not dealing with a melanin, however, but with a quite different type of pigment. Unfortunately, one is not able to decide as to whether the larvæ ultimately producing the pale *A. atra* located under littoral stones came in the first place to assume such sites in an accidental way, or were carried there and, because of some deficiency in vigor, failed to escape, or because they endured too long (or not long enough) in the larval phase and became attached at a time of day unsuitable for the operation of photic control of their movements.

The larva of *Ecteinascidia*, as I shall elsewhere describe, is normally liberated on a falling tide, and the duration of its free-swimming phase, together with its modes of response, are so adjusted to the tidal rhythm that in most cases a new individual (in this latter species, resulting in a colony) is laid down at about low-water level; the exact location of the site of attachment depends in an interesting way upon developmental changes in the phototropism of the larva. (Parallel changes have since been described by Grave, 1920, in the larva of *Amaroucium;* but the ethologic correlations, fairly clear in *Ecteinascidia*, have been as yet incompletely set forth for *Amaroucium.*) Should similar phenomena appear in the comportment of the larva of *A. atra*, some transforming individuals might become too photonegative, or might settle down at night, in either event perhaps becoming affixed in a dark cranny among stones.

4. Definite proof that darkness as such has no control over

the pigmentation of *A. atra* seems to me indicated by the finding of well-grown and densely black specimens in small caves practically shut off from the light. A few such were discovered with the aid of an electric torch in cavelets having but a small opening to the water. And from the under surface of a submerged rock, undercut in such a manner as to be completely sheltered from the sun, I later secured a group of fair-sized individuals with a quite normally black appearance.

5. *Summary.*—Pale, translucent individuals of the blue-black *Ascidia atra*, a species normally occurring in sunlit places, are found in darkened situations under stones. They resemble, in the absence of test-pigment, ascidians of other species habitually living in shaded spots. In spite of the fact that such pale *A. atra* may quickly blacken if placed in sunlight, it is pointed out that the absence of natural pigmentation cannot be regarded as due to darkness, but is probably a consequence of faulty nutrition.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 7. REGARDING THE PIGMENTATION OF STICHOPUS MŒBII.[1]

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The large holothurians of the genus *Stichopus*, which are quite abundant at Bermuda, live for the most part in fairly shallow water and upon grass-free bottoms of sandy mud. The majority of the individuals, but not all, are quite darkly pigmented. They are consequently open to the view of fishes and of other predaceous creatures, their coloration being in the great majority of cases very far from "concealing" in character. I was curious to learn if this lack of concealment was in any way related to size, or to the presence of some repugnatorial property. These holothurians do not possess Cuvierian organs.[2]

About 70 per cent. of the individuals are dorsally of a deep brown or even blackish hue, some 20 per cent. being light brown, with a distinctly yellowish cast, and the remaining 10 per cent. very pale brown with a pinkish shade. About 75 per cent. of the two kinds of lighter-colored specimens show a series of circular dark blotches, located at the bases of the scattered podia. A small proportion are irregularly mottled or blotched with areas of dark pigmentation. The pale spotted individuals are in many instances very difficult to detect, especially if located among algæ and corals on an inclined shore; on a bare mud bottom they are more easily seen. The amount of pigment in the integument bears no definite relation to age (size).

[1] Contributions from the Bermuda Biological Station for Research, No. 133.

[2] I have vainly sought for instances in which the well-developed Cuvierian organs of *Holothuria captiva* (Crozier, '15) might be seen in action (cf. Cuénot, '98). This species sometimes lives, under stones, in association with the large annelid *Hermodice carunculata*, which is armed with powerful groups of long, sharp setæ; but I have never seen evidence of disturbance of the holothurian by the annelid. The tubes of the Cuvierian organ when discharged in air and received on filter paper (or on the tongue) are never acid in reaction; apparently their sole utility is in connection with their tensile strength and adhesive power.

In view of the absolute lack of any concealing behavior practiced by these creatures, I tried at various times to feed pieces of the skin to fishes ("squirrels," yellow grunts, gray snappers, and other common shore forms, including the hamlet). Such trials were invariable unsuccessful, the flesh being refused. And, as Minchin ('92) long ago found, the intestine and respiratory trees are likewise rejected as food.

It is of interest to note that, in spite of such evidence of "unpalatability,"—which may perhaps be supplemented from Bartsch's ('17) account of the use of "holothurians" by the natives of Guam in driving *Octopus* from its "nests,"—holothurians are nevertheless occasionally seen in the rôle of hosts for erratic ectoparasites, which in some instances eat from their skin. Chatton ('09) found an ascidian attached to and growing on the integument of *Holothuria*, and I have seen a young actinian (*Condylactis*) firmly affixed to the integument of a *Stichopus*. Quite apart from such molluscan forms as *Stylifer*, one species of which I have found in considerable numbers on *S. mœbii*, I have records of an annelid (an unidentified species of *Odontosyllis*) and of a polyclad worm, *Leptoplana*, feeding on the skin of *Holothuria surinamensis;* the gut of the worm was in each of these cases filled with the bright green integumentary pigment of the host. So that it is difficult to postulate for these animals the possession of any generalized repellent secretion. Plate ('16), also, has described a polynoid and a crab, both homochromic in coloration, living upon *Holothuria atra*.

On the other hand, *Holothuria surinamensis* has on several occasions been found bearing clear evidence of injury. One specimen was picked up, lying in the open on a sandy bottom with a shallow gouge cut along the whole length of the body; in all probability it had been nipped by a crab, perhaps *Callinectes*. Later, three others were obtained, all injured in a similar way. In the absence of some repellent "flavor," it is difficult to understand why the holothurians, once bitten into in this way, should have been dropped. This particular species, as I have said, is devoid of Cuvierian organs, and in the mangrove creeks where it notably abounds it comes to the surface only at night, being

buried during the day about the roots of sea weeds. Its skin, incidentally, is covered by a slimy coating of mud-particles, tending to give it a concealing hue; whereas *H. captiva*, well equipped with Cuvierian organs, but living under stones, is of a brilliant dark green color, unobscured with mud.

In feeding tests, the flesh of *H. surinamensis* was rejected as vigorously as was that of *Stichopus*.

It seems on the whole quite possible that some repugnatorial property is present in the secretions of the holothurian skin. The repellent power of the skin of the nudibranch *Chromodoris zebra* has been amply demonstrated (Crozier, '16, and subsequent work), yet I have found ostracods (*Cypris*) creeping freely over its skin; and while the blue-black *Ascidia atra* is rejected, whole or in fragments, by fishes I have tested, certain polyclads, nevertheless, feed freely on its surface (Crozier, '17). It is not inconceivable that the slimy quality of the holothurian flesh, particularly as developed when the body wall is cut, is in itself a sufficient repellent. I have never succeeded in inducing aquarium fishes to swallow swollen pieces of gelatin, although in this case a specific repellent action seems improbable.

The adult *Stichopus* is, at all events, practically immune from the grosser kinds of attack. The only dead or injured specimens I have seen were killed by heavy rain-fall on shallow shores at low tide, or else by the sun when left by the tide on an exposed beach. The flesh of these animals is not an acceptable article of diet to fishes or to octopus, and whether this immunity be due in part to specific repugnatorial secretions, for which there is some evidence, or to some physical property of the flesh, or to the large size of the individuals, the feature which for present purposes I regard as significant is this: although efficiently protected, as it seems to be, the coloration of the species exhibits a wide range of variation, such that in some instances it might be thought "warning," in others decidedly "concealing." The conclusion is difficult to avoid, that the "conspicuous" type of pigmentation is only associated with immunity from attack in a secondary, and in a sense an accidental, manner. The flesh of the paler, spotted, and as I believe, on the whole better concealed,

individuals, is no more acceptable as food than that of the blackish specimens. The different degrees of pigmentation are determined, so far as one may judge, by quantitative differences in the capacity for producing the single type of pigmentary substance concerned. So far as available evidence permits one to say, it is as reasonable to hold that the immunity of *Stichopus*, however achieved, permits its pigment-forming power to develop unhampered by selective restraint, as to believe that a kind of "warning" coloration is being elaborated selectively. In this respect the diverse types of coloration found in the efficiently protected snail *Onchidium* (Crozier and Arey, '19) provide an interesting parallel. And in *Chromodoris* (Crozier, '19), as I shall subsequently describe at length, the evidence is much more decisively of the same import. Questions of this sort, particularly as concerned with the matter of so-called "warning" coloration, have been, in my belief, on the whole so inadequately dealt with that even partially complete observations seem worthy of presentation.

Summary.—The coloration of *Stichopus mœbii* is quite variable, in some individuals probably of a concealing tendency, in others, the majority, of a sort which makes these animals in nature pronouncedly conspicuous. Perhaps conditioned by the bulk of the animals, possibly by the presence of a repugnant quality of the flesh, such as leads to the rejection by fishes of fragments offered as food, a decided immunity from gross attack is, at all events, achieved. There is no more reason to regard the conspicuous type of pigmentation as a warning agent than there is to consider it the unchecked expression of innate metabolic activities.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 8. CONCERNING "MEMORY" IN ACTINIANS.[1]

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By a number of writers (cf. Parker, '17) it has been believed that certain animals of intertidal habitat may continue for a time to exhibit rhythmic movements, either of locomotion or of opening and closing, when they have been removed to aquaria and maintained under more or less constant conditions, and. further, that such periodic movements may be synchronous with the rise and fall of the tide. Unfortunately, many of the instances brought forward, *e.g.*, among the actinians, have been found upon closer scrutiny to yield no real evidence of a persisting "memory" of tidal events.

One of the commoner Bermudian sea-anemones, the red *Actinia bermudensis* Verr., occurs abundantly in caves along the shore and in other mud-free situations open at times to a moderate surf, but almost always between the tide lines. When the tide falls, the actinians retract the tentacles and constrict the column-sphincter. Commonly these sea-anemones hang upside down from the under faces of stones, but they are also abundant upon tiny ledges in the walls of small caves—in any event, occurring in situations such that they are rarely left in a tide pool, but are, on the contrary, freely exposed to the atmosphere by the retreating tide.

The much larger actinian *Condylactis passiflora* usually lives beneath low tide, with the column imbedded among stones; at extremely low tides, however, some individuals may be left a little distance out of water, and it is noteworthy that these do not retract the tentacles and contract the column, but instead hang down limply with the tentacles extended and the column passively stretched by the weight of the fluid in the interior cavity.

[1] Contributions from the Bermuda Biological Station for Research, No. 134.

The correlation, therefore, between a normal intertidal habitat and the practice of so contracting the body as to conceal the tentacles, decrease the surface for evaporation, and present a smoothly rounded exterior surface, is decidedly worthy of study. A variety (*prunicolor* Verr.) of *A. bermudensis* was found in bays on the south shore of Bermuda not under stones, but in small tide pools open to the sun, and also within the tidal zone. This variety is of a brownish hue, whereas the ordinary *A. bermudensis* is deep red. The color difference, apparently the only feature separating the two forms, was constant, and was even seen in young taken from the gastrovascular cavities of adults; the young of the red forms were always pink or scarlet, those of the brownish forms a light prune-color. The occurrence of such color differences has also been noted in related forms. Elmhirst and Sharpe ('20) state that the deep red *Actinia equina* becomes brown in the laboratory, and that the change "breeds true"—a remarkable observation, if correct, and one which should be followed up; it corresponds well with the conditions known in *A. bermudensis*. The red pigment of the latter species, readily extracted by acetone, is changed to a brownish hue by sunlight. The brownish anemones close up in the usual manner when the tide falls.

A. bermudensis reproduces "viviparously" throughout the year. Experiments could therefore be made with young actinians—"born" in the laboratory or taken from the cœlenteron of adults—which had never themselves been directly exposed as free individuals to the action of the tidal rhythm.

When the adults were removed from the natural location at low water and placed in an aquarium, they immediately relaxed and the tentacles were extended. After variable periods, of several hours, in non-circulating water, they closed again, although still completely covered by water. In a group of twenty individuals which were on one occasion (in January, 1917) placed in an aquarium, the period elapsing before closure was about three hours. These particular actinians all came from very nearly the same level above low water, and since most of them after a time opened again, I was led to look for a tidal rhythm. In point of fact, however, it is more than doubtful that any

such rhythm exists. The behavior of different specimens is anything but synchronous in the subsequent periods of opening and closing, and there is no tidal periodicity evident,—except perhaps in the matter of remaining expanded for about three hours after being immersed in water. But this interval is much the same regardless of the period of antecedent exposure; for specimens taken from the rock just after being left bare by the receding water are in this respect indistinguishable from those removed at the natural termination of the interval of exposure. After a sea-anemone has contracted, in still water, it may remain so for days, or it may after a time open "spontaneously." At any time it may be caused to expand by a faint local agitation of the water in its vicinity. In this behavior *A. bermudensis* was very similar to *Metridium* as described by Parker ('17). Sea-anemones attached to the rocks could always be caused to open by splashing over them a small amount of water; in this kind of response is to be seen the explanation of a sort of "anticipatory" expansion sometimes seen on the shore, wave-dashed droplets of spray being sufficient to induce some relaxation of the column-sphincter before the animals are actually covered by the sea.

Young *A. bermudensis* "born" in the laboratory aquaria exhibit precisely the same phenomena. In a group of six, one or two individuals may be found at any time tightly contracted, the others being in various degrees of expansion. But this behavior is not synchronous. One such group was rather carefully watched at intervals during 3 weeks. No rhythm of contraction was detectable in the individual behavior of these animals. Nevertheless, if they be taken from the water for a short time and then replaced, such young individuals promptly open and remain extended for about as long as do the adults under similar treatment, but after this they contract for variable periods.

Hence the rather fixed interval of expansion consequent upon immersion in sea water, although it agrees fairly well with the period of natural submergence, can hardly be ascribed to the establishment of a tidal habit; for the young actinians inexperienced in the matter of tides go through the same performance

as the adults. Of course, it might be supposed that during the period of low water changes in oxidation or the accumulation of metabolites within the cœlenteron might rhythmically influence the behavior of the embryos and juveniles. But the important point is the significance of purely mechanical stimuli, derived from motion of the water, for the relaxation of the column-sphincter. In a contracted sea-anemone, small or adult, expansion could always be secured by gentle movements of a glass rod in the nearby water. The necessary conclusion is that in the absence of such mechanical stimulation the actinian contracts; the latent period for this response is rather long. There is no evidence of tidal "memory."

Summary.—The behavior of *Actinia bermudensis* is considered with reference to its intertidal habitat. It is likely that the habit of contracting when uncovered by the sea is important for the maintenance of the species between tidal limits, but there is no evidence for persistence of the rhythm of contraction and expansion in the absence of the tidal rise and fall. After a period of about three hours in still water, the actinians close; they can at any time be caused to expand by agitating this water. Young individuals, never directly exposed to the tidal cycle, behave in the same manner.

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